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THE SPOROGENES TEST AS AN INDEX OF THE CONTAMINATION OF MILK.

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PRESENT STATUS OF THE SPOROGENES TEST.

The sporogenes test is based on the characteristic milk reaction produced by certain anaërobic spore-forming bacteria which are widely distributed in nature and which are particularly common in fecal material.

Numerous names have been given to anaërobic bacteria that give the typical milk reaction, some of which are the following: *B. enteritidis sporogenes* (Klein); *B. aerogenes capsulatus* (Welch), synonym *B. welchii* (Migula); *B. perfringens* (Veillon and Zuber); and *B. Saccharobutyricus immobilis* (Schattenfroh and Grassberger). It is generally believed that the organisms bearing these names are either identical or very closely related species.

In milk, under anaërobic conditions, these organisms produce what is known as the "stormy" fermentation. In a characteristic reaction the casein is coagulated and the curd torn by gas within 24 hours at 37° C. The whey is usually quite clear and the odor of butyric acid is noticeable. When the milk in a test tube is covered with a paraffin plug the latter is usually forced up almost to the top of the tube and sometimes entirely out of the tube.

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English bacteriologists have made use of this characteristic reaction, both in water and milk analysis, in order to detect the presence of *B. enteritidis sporogenes*, which in their opinion indicates contamination by fecal material. The test has consequently become known as the sporogenes test. Therefore, in this paper the name *B. enteritidis sporogenes* will be used in designating the organism causing the stormy reaction, except when direct reference is made to publications in which different terms are used. The organisms giving this test should not be confused with *B. sporogenes* of Metchnikoff, which gives an entirely different milk reaction.

Whether the bacteria which show the sporogenes test are identical makes little difference provided their distribution in nature is about the same. They are known to be present in fecal material, in miscellaneous foodstuffs, cattle feed, soil, and water. Since they are present in cow feces, it is natural to assume that their presence in milk may be used as an index of contamination by manure. If they are present in feeds and soil to the same extent as in manure, their presence in milk may be rather an index of the general condition of cleanliness of production.

The obvious advantage of using the spores of *B. enteritidis sporogenes* as an index of contamination lies in the fact that pasteurization does not destroy them, and therefore does not interfere with the determination of their presence in milk. Furthermore, they are not believed to vegetate in milk under normal conditions, and vegetative cells do not sporulate in the presence of the fermentable sugar in milk; so the sporogenes test with both fresh and old milk, provided it is still sweet, should give the same results. The test has consequently become known as a "nonmultiplying test." Should it prove to be reliable in gauging the cleanliness of production and be relatively accurate, it would be extremely valuable in milk-control work.

It has not been difficult to find spores in milk which give the characteristic stormy reaction. Flügge (4)[1] in 1894 isolated anaërobic butyric-acid bacteria from milk. In 1897 Klein (8) examined milk in London for spores of *B. enteritidis sporogenes* and found them in 8 of the 10 samples examined. Since then they have been found in milk by various investigators, among whom are Savage (12), Houston (7), Barthel (1), Brown (2), Simonds (14), Ritchie (11), Pryor (9), Weinzirl (15), Shippen (13), and Ford (5). Probably spores of these anaërobic bacteria can be found in all milk if large enough amounts are examined.

There seems to be a difference of opinion among different investigators as to the value of the sporogenes test in indicating manurial contamination and the general conditions of cleanliness in produc-

[1] The numbers in parentheses refer to "Literature cited," at end of bulletin.

tion. Savage (12), who was among the first to apply the test for this purpose, and who has studied the test probably more than any one else, realized its limitations. He states that the test does not show so close an agreement with the cleanliness of farm conditions as does the estimation of *B. coli.* On the whole, however, he thought that the sporogenes test might be of considerable value in estimating pollution, especially in market milk. Ritchie (11) concluded from his results that the sporogenes test was of little value. He obtained no correlation between the number of positive reactions and farm conditions. Barthel (1) was of the opinion that there never was a direct relation between the hygienic quality of milk and the presence of strict anaërobes. On the other hand, Weinzirl and Veldee (15) have used the test as a means of determining the manurial pollution of milk and believe it to be of distinct value.

There is nothing new about the sporogenes test so far as its general application is concerned. It has been known for a long time, but it has not been given a thorough trial under controlled conditions of production. From past results the test gives promise of being too valuable to discard, yet it is too uncertain at present to use without knowing its limitations.

It is the purpose of this paper to present some results obtained with the sporogenes test on milk produced under controlled conditions.

THE SAVAGE METHOD.

Savage (12) first examined 1 c. c., 10 c. c., and 20 c. c. of milk, the smallest quantity being added to a tube of freshly sterilized whole milk, while the other quantities were placed in empty, sterile test tubes. The milk was heated to 80° C. for 10 minutes, then cooled and incubated under anaërobic conditions at 37° C. After 48 hours the tubes were examined for the typical stormy reaction. These quantities were found to be too wide apart to yield a satisfactory estimation of the number of spores, so the following method was advocated. Small, narrow tubes, about 4 inches by $\frac{1}{4}$ inch, were used in batches of 10. The tubes were sterilized and 2 c. c. of milk added to each, making a total of 20 c. c. in the 10 tubes. The tubes were heated, incubated, and examined as mentioned above. Each tube which showed a typical stormy reaction was recorded as 1. Thus, if three tubes showed the reaction, the result was recorded as 3.

Savage set the following arbitrary standard, which he says can not be considered a rigid standard:

0 or 1 tube positive=good milk.

2, 3, or 4 tubes positive=unsatisfactory milk.

5 or more tubes positive=bad milk.

A number of samples of milk produced under dirty conditions have been examined by the Savage 10-tube method. His method was

varied slightly in that sterile paraffin was poured into each tube after heating. This formed a plug over the milk, and it was not necessary to place the tubes under anaërobic conditions.

It was considered advisable to try the test on milk produced under conditions that would represent the worst grade of milk which might be encountered under commercial conditions. In order to do this, four cows were placed in a small barn which had been used for similar experimental purposes. The loft above the cows was composed of narrow boards laid from 1 to 2 inches apart. Hay and cobwebs hung down from these openings. The walls were soiled with manure and dirt. All the cows were allowed to become dirty and their udders and flanks were more or less covered with partly dried manure. The manure was removed from the floor only twice a week. Open pails, not sterilized, were used for milking.

To show the relation between the Savage sporogenes test and the milk, the sediment from 1 pint of milk, the total count, and the result of the Savage test are shown in Plates I, II, and III. In the upper right-hand corner of each square is a number designating the number of tubes showing a positive sporogenes test out of the 10 tubes used for each sample. Keeping in mind the arbitrary standards set by Savage (that is, 0 or 1 + = good milk, 2, 3, or 4 + = unsatisfactory milk, 5 or more + = bad milk) the results are interesting. It will be noted that according to this test the milk from Samples 1 to 35, inclusive, would be called good milk. It is believed that the sediment disks and counts make further discussion unnecessary. Particular attention is called to the difference in sediment between Sample 1 and Samples 19 and 20. None of the three showed a positive test by the Savage method. On Plate III samples from 36 to 52, inclusive, would be classed as unsatisfactory by the Savage sporogenes test. No one would dispute this statement, although many were no worse than those called good on Plates I and II. Samples 53, 54, and 55 are classed as bad by the test, yet they are no worse than some called good.

It is further evident from the results shown on the plates that there is no relation between the sporogenes test as used by Savage and the total count. In this connection it may be noted that the milk examined was fresh milk. Savage also found very little relationship between the test and the total count.

The question naturally arises in connection with the sporogenes test as to the accuracy of the test itself. Will a number of tests with a given sample of milk show the same results? To answer this question, 5 sets of 10 tubes each, with 2 c. c. of milk in each tube, were prepared from a sample of milk. In other words, the Savage method was applied 5 times to the same sample of milk. From the results of

14 samples of milk examined in this manner it is evident as shown in Table 1 that there may be a considerable variation in the results obtained from a given sample.

TABLE 1.—*Variation in the Savage sporogenes test, showing number of positive tubes of 2 c. c. each after incubating for 48 hours at 37° to 40° C.*

Sample.	Number of positive tests.					Range in number of positive tests.
	Set 1.	Set 2.	Set 3.	Set 4.	Set 5.	
1	3	5	6	3	2	2 to 6
2	4	0	2	1	3	0 to 4
3	2	2	1	0	2	0 to 2
4	1	1	1	0	0	0 to 1
5	1	0	0	1	1	0 to 1
6	1	2	0	1	2	0 to 2
7	2	1	2	2	1	1 to 2
8	1	2	2	0	2	0 to 2
9	1	0	0	0	0	0 to 1
10	4	4	1	3	3	1 to 4
11	9	8	5	6	7	5 to 9
12	6	8	5	6	6	5 to 8
13	7	6	3	7	6	3 to 7
14	9	8	9	7	9	7 to 9

The sample of milk in every case was shaken thoroughly to give as equal a distribution of spores as possible. The 2 c. c. samples used in the sporogenes test were removed in two different ways: (1) Ten 2 c. c. samples were removed from the sample of milk by means of a sterile 2 c. c. pipette; (2) ten 2 c. c. samples were removed from the sample of milk by means of a sterile 10 c. c. graduated pipette, successive 2 c. c. portions being delivered for each sample. It was found that this variation in removal of the 2 c. c. samples had no effect on the results of the sporogenes test.

The effect of the variations in the numbers of positive tubes and different sets of tubes of milk from the same sample of milk is obvious when one attempts to grade the milk by the Savage method. For example, in Table 1, Samples 2, 3, 6, 8, and 10 would be graded as either *good* or *unsatisfactory*, according to the results of the sporogenes test and the Savage arbitrary standards. This is shown more clearly by consideration of the results obtained with Sample 2. Each set of 10 tubes represents a complete sporogenes test according to the Savage method. In Set 2 there were no positive tests and in Set 4 there was one; therefore, the milk would be graded by either of these tests as good. In the other sets there were 2, 3, and 4 tubes positive, which according to the standards would necessitate calling the milk unsatisfactory.

Two reasons at least may be designated as contributing causes for the variations in the sporogenes test: First, uneven distribution of the spores in the milk; and, second, lack of development of the characteristic stormy reaction on which the test is based. Savage (12) mentions the fact that the lack of an even distribution of spores is a

serious drawback to the utility of the test, while Simonds (14) found that a failure to get the stormy fermentation does not necessarily mean a lack of growth of the organisms giving the test. This he believes from his experiments is due to not having the correct degree of anaërobiosis in the milk tubes.

How far the lack of development of the characteristic stormy reaction influences the assumption of an unequal distribution of spores is difficult to determine. As long as these variations in the sporogenes tests continue, the value of the test is materially lowered, and the reason for the variation is of little importance except that if it were definitely known some remedy might be found. This defect in the sporogenes test applies not only to the Savage method but also to the other methods mentioned in this paper.

THE WEINZIRL METHOD.

A method of determining manurial pollution of milk in which the sporogenes test is used has been suggested by Weinzirl (15). The method of making the test is essentially the same as the first one used by Savage, although the results were interpreted in a somewhat different manner. The method of Weinzirl consists in using 5, 10, and 15 c. c. samples of milk from each sample which is to be examined. The milk is heated to 80° C. for 10 minutes and melted paraffin poured on the milk to make a layer one-eighth inch or more in thickness. The tubes of milk are then cooled and incubated at 37° C. From his examination of cow manure, Weinzirl calculated that there were probably 10,000 sporogenes per gram of manure in the partially dried condition in which it usually enters milk. Based on this figure, he estimated the amount of manure in milk as follows: A positive reaction in the 5 c. c. tube indicates 1 gram of manure in 50 liters of milk; a positive reaction in the 10 c. c. tube indicates 1 gram of manure in 100 liters of milk; and a positive reaction in a 15 c. c. tube indicates 1 gram of manure in 150 liters of milk.

To determine the value of Weinzirl's method, 48 samples of milk were examined which were produced under dirty conditions. The same conditions of production prevailed as those previously described under the Savage test. The results of the work are presented in Plates IV and V, on which are shown sediment disks from each pint sample of milk examined, together with the results of the sporogenes test as performed by Weinzirl and the total bacterial count. Fresh milk was examined in all cases. In the upper left-hand corner of each square is shown the result of the sporogenes test. Figures 5, 10, and 15 represent the respective number of cubic centimeters of milk used, and opposite each is a positive or negative sign. A positive sign indicates a "stormy" fermentation, and a negative sign the absence of the characteristic reaction.

In order to show the value of the Weinzirl test, the samples of milk have been grouped on the basis of the test in accordance with his arbitrarily assumed standards. Weinzirl's standards were as follows: One gram of manure in 50 liters of milk is excessive; not more than 1 gram in 100 liters of milk is fair; and not more than 1 gram in 150 liters represents good market milk.

On the basis of this classification, all the samples represented by sediment disks in Plate IV would be classed as good milk. Undoubtedly such a classification is incorrect. Particular attention is called to the differences in the quantity of manure among samples showing the same results with this sporogenes test. Compare, for example, Samples 1 and 15. In neither case were any positive reactions recorded.

The sediment disks on Plate V were made from milk which would be classed as fair or as having an excessive quantity of manure, according to Weinzirl. The last three samples are the only ones which would be considered to have an excessive amount of manure. It is evident that, with the exception of Sample 36, the rest of the samples at least do not represent good milk, and are certainly no better than the fair milk as classified by the Weinzirl test. Many of these samples of so-called fair milk, however, were evidently much better than some of those classed as good milk.

As in the Savage method, there is also no definite relation between the Weinzirl sporogenes test and the total bacterial count. Particular attention is called to the variation between the test and the amount of sediment. This is especially striking in the case of Samples 15 on Plate IV and 36 on Plate V. Sample 15 had an excessive quantity of manure showing a negative sporogenes test, while Sample 36 had a small amount of sediment and yet gave a positive reaction in the 5 c. c. tube. Positive tests among the three quantities of milk used seem to show no consistent behavior. If a sufficient number of spores are present in the milk to give a positive test in the 5 c. c. tube, the 10 and 15 c. c. tubes should also be positive. If the 10 c. c. tube is positive, the 15 c. c. tube should also show the reaction, but such is not always the case. These variations probably are due either to an uneven distribution of spores or to their failure to develop a characteristic stormy reaction. Weinzirl, of course, merely assumed an arbitrary standard for grading milk on the basis of the sporogenes test, but whatever standard is accepted it would not change the lack of correlation between his methods of making the sporogenes test and the quantity of manure actually in the sample.

DEFECTS IN THE SPOROGENES TEST.

Besides the variations in results obtained by repeating the sporogenes test on the same samples of milk, the test is defective because it

is not positively known how the spores of the organisms giving the test gain entrance to milk. This fact naturally decreases the utility of the test. Savage recognizes this condition of affairs, and in weighing the value of the test rightly assumes that they might gain entrance to milk from other sources, and thus would represent contamination by material not so undesirable as manure.

Weinzirl seems to have overlooked the possibility of the entrance of the spores of *B. enteritidis sporogenes* from sources other than cow manure. He makes a further assumption, which from our results seems to be incorrect—that of giving an average figure which can be safely set up as being the number of spores per gram of manure. He estimates that cow manure as it enters milk contains the probable average of 10,000 spores per gram. He found that in moist cow manure there may be fewer than 730 or more than 14,300 per gram. This is a decidedly higher figure than was obtained by Savage, who estimated from 10 to 10,000 per gram. As is shown in Table 2, our figures for the number of spores of *B. enteritidis sporogenes* in cow feces are much lower than those obtained by Weinzirl, and agree closely with the results of Savage. In fact, our figures, on the whole, are even lower than those of Savage. The manure examined consisted of samples ranging from fresh to nearly air dry, the most of them, however, being decidedly moist.

TABLE 2.—*Number of spores of B. enteritidis sporogenes in cow manure and feed.*

Sample No.	Cow feces.	Feed.		Sample No.	Cow feces.	Feed.	
		Silage.	Grain.			Silage.	Grain.
	Spores per gram.	*Spores per gram.*	*Spores per gram.*		*Spores per gram.*	*Spores per gram.*	*Spores per gram.*
1	40	60	....	13	140	....	30
2	50	[1] 0	....	14	100	....	20
3	200	400	....	15	140	....	40
4	600	800	....	16	600	....	35
5	20	20	....	17	20	....	[1] 0
6	50	55	....	18	240	....	15
7	600	30	....	19	40	....	20
8	60	15	....	20	20	....	30
9	800	80	....	21	20	....	100
10	1,000	40	....	22	140	....	20
11	800	60	....	23	....	....	40
12	1,000	80	....	24	....	....	200

[1] 0–$\frac{1}{10}$ gram.

The average spore content of 22 samples representing different cows from herds in widely scattered parts of the country was estimated to be 304 per gram. The number of spores was estimated by inoculating several series of 5 tubes of sterile milk each with a different dilution of manure in sterile water. The tubes were then heated to 80° C. for 10 minutes, then cooled and incubated. From

the number of stormy reactions and the dilution the number of spores in the manure was estimated. Assuming that the manure as it enters milk contains only one-fourth as much moisture as samples of moist feces, and multiplying, therefore, the average spore content by four, it may be assumed from the results that the manure as it enters the milk contains approximately 1,200 spores per gram. When this average figure is compared with the 10,000 obtained by Weinzirl it is apparent that there must be an extremely variable number of spores of *B. enteritidis sporogenes* in cow feces. Even if it were possible to assume that these spores could gain entrance into milk from cow manure only (without definite proof this assumption can not safely be made) their variable number in this material would decidedly interfere with the accuracy of the test. In the light of these facts it is probably possible to explain the discrepancies that exist between the test and the manurial content of the milk.

Spores of the organism under consideration are known to be widely distributed in nature. They are found in waters and soils, and, as is shown in Table 2, they are present in considerable numbers in silage, grain, and mixed feeds. How many spores may gain entrance into milk from these sources is of course problematical, although on the whole it seems probable that they are introduced in the greatest numbers through manure.

It seems apparent from the results of other investigations and of our experiments that if the sporogenes test is to be of any definite value its greatest possibility lies in the relation which the test bears to the general conditions of production.

ATTEMPTS TO IMPROVE THE CHARACTERISTIC STORMY REACTION.

Before going further with the sporogenes test it was considered desirable (1) to reduce if possible the number of doubtful reactions caused by peptonizing faculative anaërobes and (2) to hasten and make more dominant the growth of *B. enteritidis sporogenes* by adding to milk certain substances likely to promote more rapid growth of the organism.

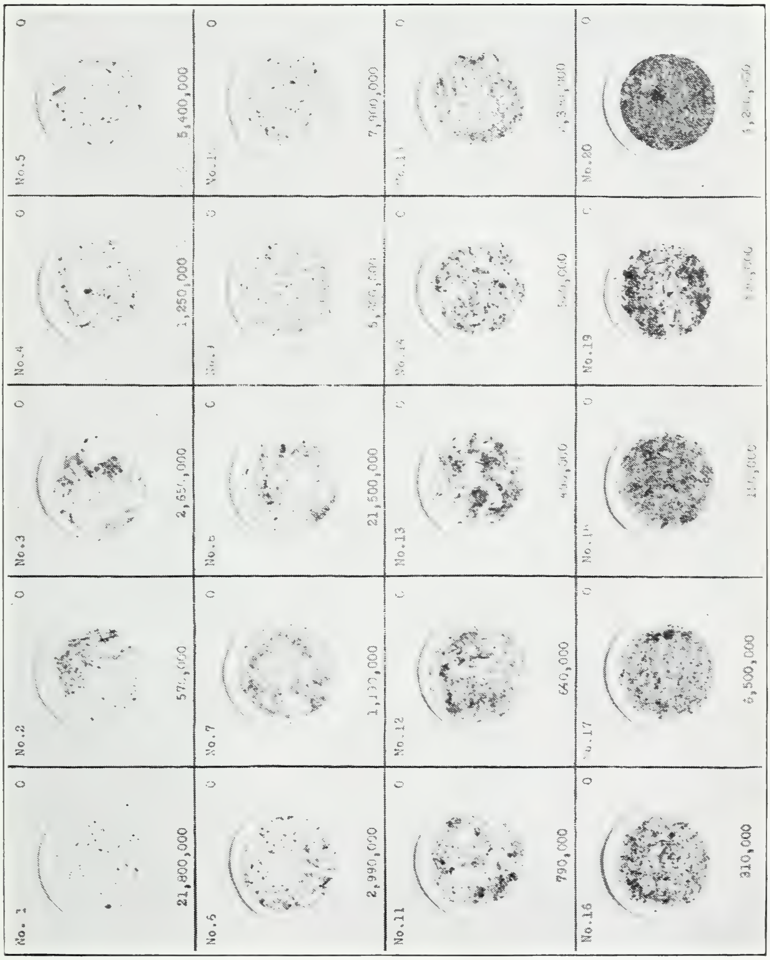
To eliminate peptonization, attempts were made to utilize the selective action of gentian violet. Churchman (3) has shown that gentian violet in a dilution of 1 to 100,000 of medium prevented the growth of *B. subtilis* while it permitted the growth of *B. welchii.* Dilutions of 1 to 50,000 and 1 to 100,000 were tried, the necessary amount of a stock solution of 1 to 1,000 being added previous to heating the milk tubes. It was found that the proportions of 1 to 50,000

largely eliminated peptonization and made the stormy reaction more clear-cut, but at the same time reduced the number of positive tests, probably because of an inhibiting action on *B. enteritidis sporogenes.* With a strength of 1 to 100,000 the gentian violet caused no reduction in the number of positive tests, but also had no effect in eliminating peptonization in samples containing facultative anaërobes capable of producing this reaction. While peptonization in occasional tubes is not a very serious matter, it tends to create difficulty in determining whether a tube shows a typical stormy fermentation. The use of gentian violet proved to be of no assistance in this connection.

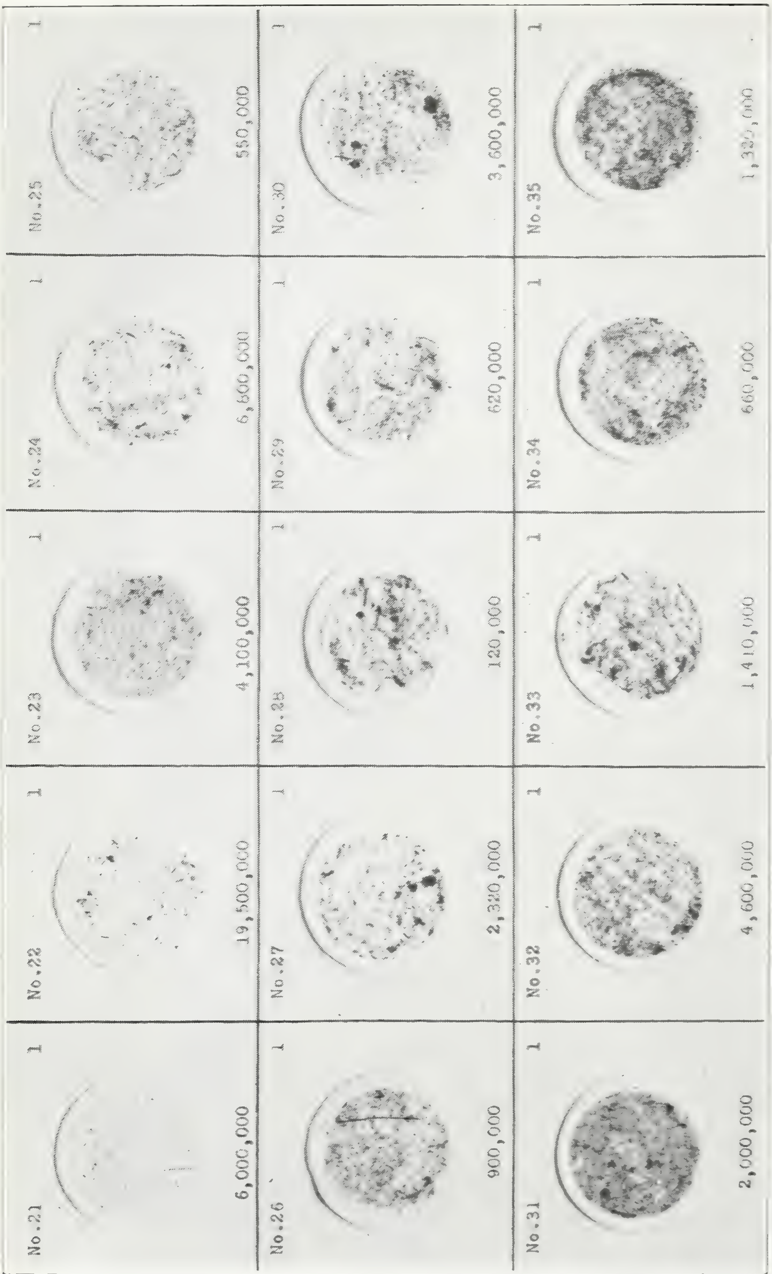
Henry (6) made use of alkaline egg albumen in connection with the development of *B. welchii* on solid media and obtained beneficial results. It was thought, therefore, that it might be possible to use this in connection with the sporogenes test in order to stimulate the growth of these organisms. The alkaline egg medium as given by Henry is as follows:

To the whites of two eggs 4 c. c. of N/1 NaOH is added and the mixture heated at about 95° C. for 1½ hours. Solution is then made up to 330 c. c. volume with water and then filtered and sterilized. When using the sporogenes test with 10 tubes of 2 c. c. each of milk, 1 c. c. of sterile, alkaline egg solution was added to each tube before the milk. The tubes were then heated and carried through the test in the usual manner. In some samples of milk it was found that when alkaline egg was used the number of positive tubes in the set of 10 was considerably increased. Quite often the reaction appeared more quickly than in the tubes without the egg solution and as a rule the reaction was more vigorous. This likely improvement in the test did not follow consistently, however, in all samples, and it was found that the advantage gained did not compensate for the additional trouble of using the alkaline egg solution.

The effect of the addition of peptone on the sporogenes test was also tried. In this experiment 10 tubes, each containing 10 c. c. of milk, were used instead of the 2 c. c. amounts used by Savage. Two series of tubes of 10 c. c. each were prepared, one series containing approximately 5 per cent peptone and the other containing none. One c. c. of a sterile 5 per cent solution of peptone was placed in each tube of the peptone series, to each of which was then added 10 c. c. of milk. Nineteen samples of milk were examined on this comparative basis and the results indicated that in some cases the addition of peptone caused an increase in the number of positive tubes and also a more vigorous reaction. In other samples the number of positive reactions was lower in the milk with peptone than in that without. On the whole, the results indicated that there was no particular advantage in the addition of the peptone solution.



SEDIMENT DISKS FROM PINTS OF MILK PRODUCED UNDER DIRTY CONDITIONS, WITH UTENSILS NOT STERILIZED, SHOWING RESULTS WITH SAVAGE'S SPOROGENES TEST AND THE TOTAL COUNT.



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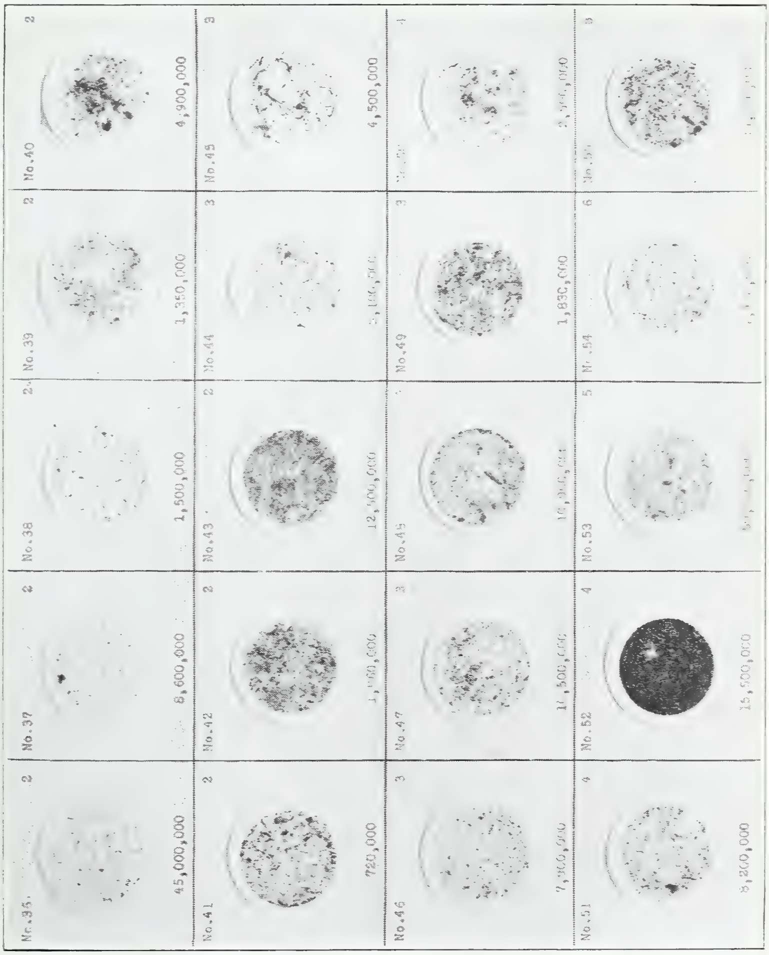

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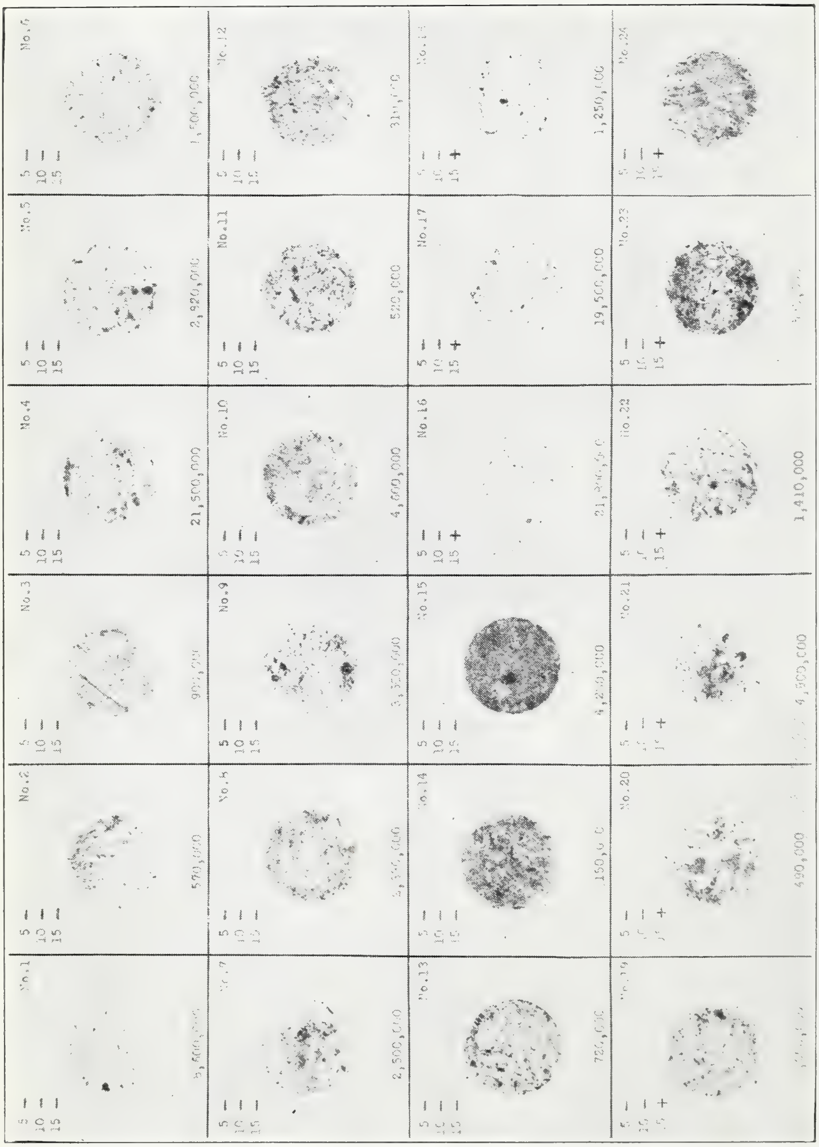
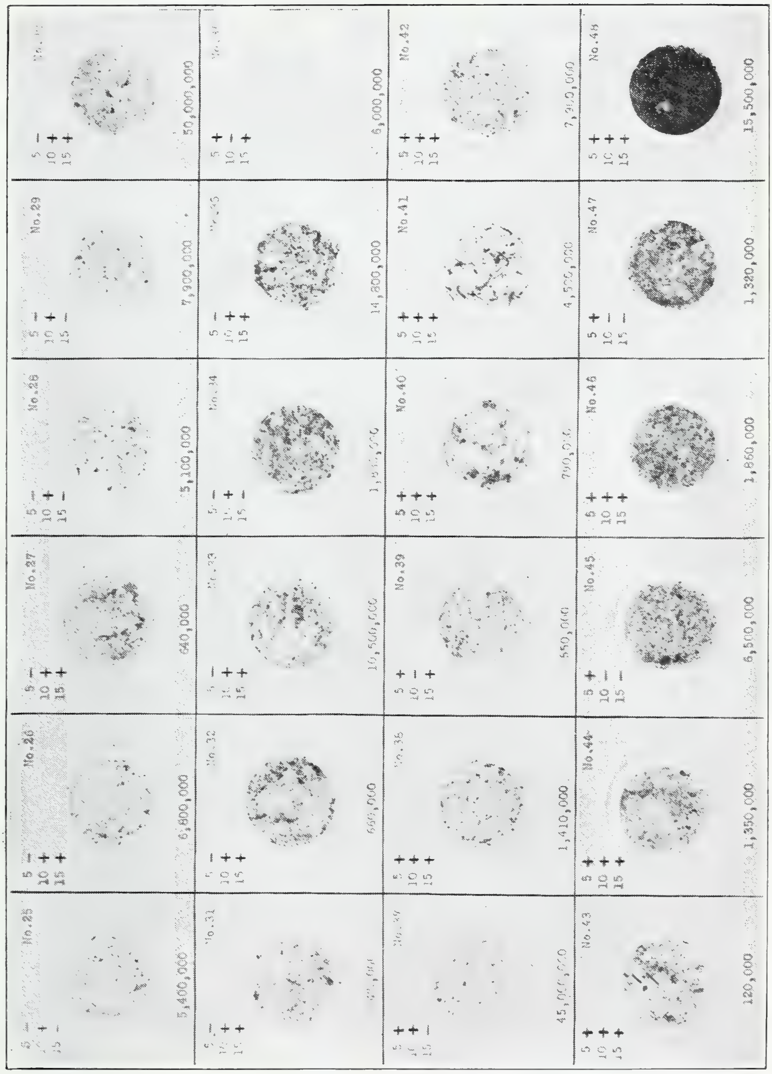


SEDIMENT DISKS FROM PINTS OF MILK PRODUCED UNDER DIRTY CONDITIONS, WITH UTENSILS NOT STERILIZED, SHOWING RESULTS WITH WEINZIRL'S SPOROGENES TEST AND THE TOTAL COUNT.



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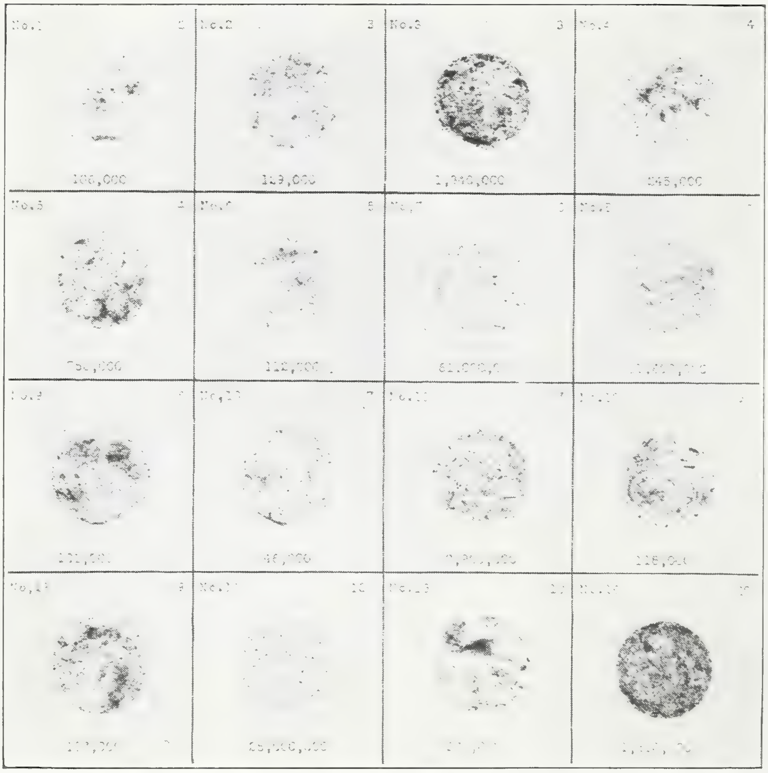

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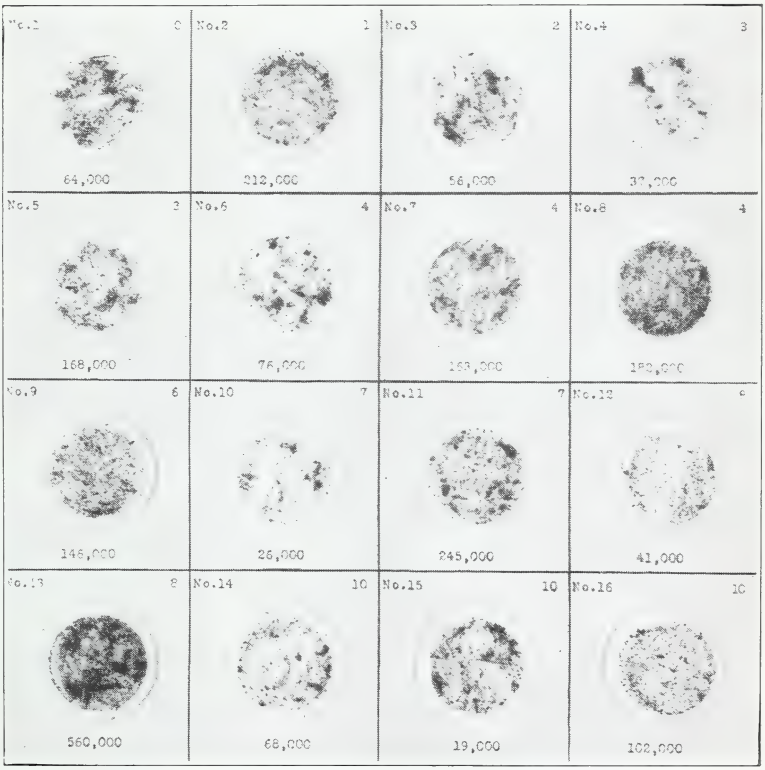

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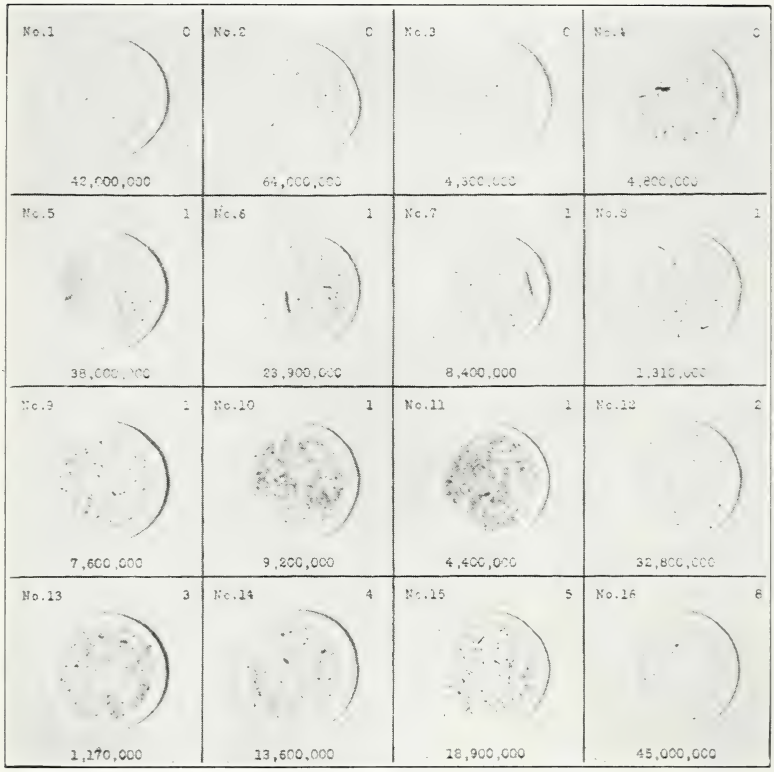

SEDIMENT DISKS FROM PINTS OF MILK PRODUCED UNDER CLEAN CONDITIONS, WITH UTENSILS NOT STERILIZED, SHOWING RESULTS OF THE SPOROGENES TEST AND THE TOTAL COUNTS.

USE OF 20 C. C. AMOUNTS OF MILK IN THE SPOROGENES TEST.

While working with the Savage test where 2 c. c. amounts of milk were used in 10 tubes, it seemed apparent that if the test was to be used in the most efficient manner its range would have to be increased so as to allow a greater difference, if possible, between good and bad milk. It seemed probable that this might be accomplished by using 10 c. c. or 20 c. c. quantities of milk.

A series of tests was therefore made, using 2 c. c., 10 c. c., and 20 c. c. of milk, produced under the dirty conditions previously described. The results in Table 3 show clearly the value of larger samples of milk. They are arranged in the order of the number of positive reactions in the 2 c. c. samples, and the corresponding results with 10 and 20 c. c. are placed opposite. It should be kept in mind that all the milk examined was produced under extremely dirty conditions and contained an abnormal quantity of manure in many cases. In other words, the milk represented the worst grade that would ever be encountered in commercial conditions.

TABLE 3.—*Comparison of number of positive reactions when different quantities of milk were used.*

Sample No.	Sets of 10 tubes, 2 c. c. each.										Sets of 10 tubes, 10 c. c. each.										Sets of 10 tubes, 20 c. c. each.									
1	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
2	−	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	−	−	−	−	−
3	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	−
4	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−
5	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
6	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−
8	−	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−
9	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	−	−	−
10	+	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	+	−	−	−
11	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−
12	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−
13	+	−	−	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	+	−	−	−
14	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−
15	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	−	−	−
16	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−
17	+	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	−
18	+	+	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	−	−	−
19	+	+	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−	+	+	+	+	−	−	−	−	−	−
20	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	−	−	−
21	+	+	+	−	−	−	−	−	−	−	+	+	+	−	−	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−
22	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	−	−	−	−
23	+	+	+	+	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	−	−
24	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−	+	+	+	+	−	−	−	−	−	−
26	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+	−	−
27	+	+	+	+	+	−	−	−	−	−	+	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+	+	−
28	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	−	−	+	+	+	+	+	+	+	+	+	+
29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Keeping this fact in mind, it is interesting to observe that more than one-half of the samples in which 2 c. c. of milk were used showed either none or only one positive reaction. The first 8 samples showing no positive reactions could not be differentiated by means

of this test from the highest grade of milk that could be produced. The results with the 10 c c. amount of milk were not found to be so satisfactory as the 20 c. c., because during the examination of certified milk a positive test was rarely encountered with the smaller amount.

It was felt that an amount of milk should be used which if possible would show at least one positive reaction in a set of 10 tubes with milk produced under the cleanest conditions, and would show also a large number of positive reactions with milk produced under extremely dirty conditions. The results in Table 3 show that 2 c. c. amounts of milk do not meet this requirement, for with dirty milk 8 out of 30 samples gave no positive sporogenes test. In the same dirty milk there was a greater tendency for a larger number of the 10 tubes with 20 c. c. each to show the characteristic stormy reaction. Even with the 20 c. c. quantities the results were not so uniformly high as could be desired, but this amount of milk possessed the added advantage of being about the smallest amount which could be used to show positive reactions in milk produced under the cleanest conditions.

THE SPOROGENES TEST IN RELATION TO MILK PRODUCED UNDER EXTREME CONDITIONS OF CLEANLINESS AND OF FILTH.

It was evident that if the sporogenes test could not differentiate between samples of milk produced under clean and dirty conditions there could be no hope of the test's being of value in milk-control work. To determine this point the sporogenes test, using 10 tubes with 20 c. c. of milk, was applied to 24 samples of certified milk and 18 samples of milk produced under the dirty conditions previously described. The results in Table 4 show that the test with 20 c. c. of milk to each tube can be used with milk produced under extremes in conditions. About 50 per cent of the samples of certified milk showed no positive reaction, but of the remaining 13 samples 7 showed 1 positive tube, 5 showed 2 positive, and 1 showed 4 positive.

TABLE 4.—*The sporogenes test with milk produced under clean and under dirty conditions.*

CERTIFIED MILK.

Sample No.	Sporogenes test in sets of 10 tubes, with 20 c. c. of milk.										Positive tests.	Bacterial count.
1.....	+	+	—	—	—	—	—	—	—	—	2	3,900
2.....	+	+	—	—	—	—	—	—	—	—	2	4,400
3.....	—	—	—	—	—	—	—	—	—	—	0	4,600
4.....	+	—	—	—	—	—	—	—	—	—	1	6,500
5.....	—	—	—	—	—	—	—	—	—	—	0	8,200
6.....	—	—	—	—	—	—	—	—	—	—	0	8,600
7.....	—	—	—	—	—	—	—	—	—	—	0	8,100
8.....	—	—	—	—	—	—	—	—	—	—	0	9,900
9.....	+	—	—	—	—	—	—	—	—	—	1	10,300
10.....	+	+	+	+	—	—	—	—	—	—	4	10,500
11.....	+	+	—	—	—	—	—	—	—	—	2	10,500
12.....	+	—	—	—	—	—	—	—	—	—	1	11,000
13.....	+	—	—	—	—	—	—	—	—	—	1	12,200
14.....	+	—	—	—	—	—	—	—	—	—	1	12,600
15.....	—	—	—	—	—	—	—	—	—	—	0	12,700
16.....	+	+	—	—	—	—	—	—	—	—	2	16,700
17.....	—	—	—	—	—	—	—	—	—	—	0	18,000
18.....	+	—	—	—	—	—	—	—	—	—	1	20,700
19.....	—	—	—	—	—	—	—	—	—	—	0	28,400
20.....	—	—	—	—	—	—	—	—	—	—	0	31,500
21.....	—	—	—	—	—	—	—	—	—	—	0	31,800
22.....	—	—	—	—	—	—	—	—	—	—	0	36,600
23.....	+	—	—	—	—	—	—	—	—	—	1	40,700
24.....	+	+	—	—	—	—	—	—	—	—	2	45,000

MILK PRODUCED UNDER DIRTY CONDITIONS; COWS DIRTY; UTENSILS NOT STERILIZED.

1.....	+	+	+	+	+	+	+	+	+	—	9	33,000
2.....	+	+	+	+	+	+	+	—	—	—	7	46,000
3.....	+	+	+	+	+	+	—	—	—	—	6	101,000
4.....	+	+	—	—	—	—	—	—	—	—	2	106,000
5.....	+	+	+	+	+	+	+	+	+	—	9	107,000
6.....	+	+	+	+	+	—	—	—	—	—	5	112,000
7.....	+	+	+	+	+	+	+	+	+	—	9	118,000
8.....	+	+	+	—	—	—	—	—	—	—	3	129,000
9.....	+	+	+	+	—	—	—	—	—	—	4	245,000
10.....	+	+	+	+	+	+	+	+	+	+	10	320,000
11.....	+	+	+	+	—	—	—	—	—	—	4	750,000
12.....	+	+	+	—	—	—	—	—	—	—	3	1,340,000
13.....	+	+	+	+	+	+	+	+	+	+	10	1,810,000
14.....	+	+	+	+	+	+	+	—	—	—	7	2,900,000
15.....	+	+	+	+	+	+	—	—	—	—	6	11,600,000
16.....	+	+	+	+	+	+	+	—	—	—	7	22,500,000
17.....	+	+	+	+	+	+	+	+	+	+	10	25,000,000
18.....	+	+	+	+	+	+	—	—	—	—	6	61,000,000

An examination of the results obtained from milk produced under extremely dirty conditions shows that in general the number of positive tubes was considerably higher than in the case of the certified milk. It is true that there were some samples which showed only two or three positive results. Such samples would be difficult to place by this test. The general picture presented by the results, however, is such that a marked difference is shown in the results obtained by the sporogenes test when milk is examined which has been produced under these extreme conditions. It is evident that these results determine the limitations of the test. The number of positive reactions in a set of 10 tubes using 20 c. c. quantities of milk, therefore, must fall either within the range of the results shown in Table 4, which represent the examination of good and bad milk, or somewhere between these fig-

ures. It is apparent that there is not a very great range left to cover the results obtained in the examination of the intermediate grades of milk. From these results, however, it seems probable that the sporogenes test, using 10 tubes of 20 c. c. quantities of milk for each tube, may be of some value in determining whether milk has been produced under clean or dirty conditions. One test, however, would probably not be sufficient to enable one to make this decision. If several tests were made and the great majority of the results were either high or low, the results could be interpreted more accurately and the value of the test would be greatly improved.

It is impossible to say from these results where milk produced under fair conditions would fall according to this grouping. The tendency would be for such samples to show great variations, some falling into the clean grade, others into the dirty grade.

CONDITIONS OF PRODUCTION OF PASTEURIZED MILK AS INDICATED BY THE SPOROGENES TEST.

In connection with the results just discussed it will be of interest to see what commercially pasteurized milk showed, using this test to indicate conditions of production. This is possible, because the test is probably a nonmultiplying one, as shown by the work of Savage (12). To verify this point several experiments were conducted by holding pasteurized milk at room temperature for 24 hours. No change in the number of spores was observed other than what could be accounted for by the limitations of the test.

Since the spores are not destroyed by the usual pasteurizing temperatures and do not change in number, the sporogenes test, if it correlates with the presence of manure or with the general conditions of cleanliness in production, should, as suggested by Weinzirl, be an excellent test for determining the quality of the raw milk before pasteurization. The results which are shown in Table 5 were on the 10 c. c. and 10-tube basis, because the samples of milk were examined before the value of 20 c. c. quantities of milk was known. When examining the results it should be kept in mind that certified milk shows negative results with this quantity of milk. Consider, now, the results of the sporogenes test with dirty milk shown at the bottom of the table, and what can then be said of the conditions under which the raw milk was produced.

TABLE 5.—*The sporogenes test with milk produced under unknown conditions and milk produced under dirty conditions.*

COMMERCIAL PASTEURIZED MILK.

Sample No.	Sporogenes test in sets of 10 tubes, with 10 c. c. of milk.										Positive tests.	Bacterial count.
1	+	—	—	—	—	—	—	—	—	—	1	3,100
2	+	+	+	+	—	—	—	—	—	—	4	3,500
3	+	+	+	+	—	—	—	—	—	—	4	4,800
4	+	+	+	+	+	+	+	+	—	—	8	5,700
5	+	+	+	+	—	—	—	—	—	—	4	10,400
6	+	+	+	—	—	—	—	—	—	—	3	10,800
7	+	+	+	+	+	+	+	+	+	+	10	12,400
8	+	+	+	+	+	+	+	+	+	—	9	12,500
9	+	+	+	—	—	—	—	—	—	—	3	13,700
10	+	+	+	+	+	+	+	+	—	—	8	15,600
11	+	+	+	+	+	+	+	+	+	+	10	15,800
12	+	+	+	+	+	+	+	—	—	—	7	17,600
13	+	+	+	+	+	+	+	+	+	—	9	17,800
14	+	+	+	+	+	+	+	+	—	—	8	17,800
15	+	+	+	+	+	+	+	+	+	—	9	18,800
16	+	—	—	—	—	—	—	—	—	—	1	19,200
17	+	+	—	—	—	—	—	—	—	—	2	19,300
18	+	+	—	—	—	—	—	—	—	—	2	20,200
19	+	+	+	+	—	—	—	—	—	—	4	20,300
20	—	—	—	—	—	—	—	—	—	—	0	22,800
21	+	+	+	+	+	+	—	—	—	—	6	24,700
22	+	+	+	+	+	+	+	+	+	—	9	25,200
23	+	+	+	+	+	+	+	—	—	—	7	26,900
24	+	+	+	+	—	—	—	—	—	—	4	30,600
25	+	+	—	—	—	—	—	—	—	—	2	41,900
26	+	—	—	—	—	—	—	—	—	—	1	57,000
27	+	+	+	+	—	—	—	—	—	—	4	80,000
28	+	+	+	+	+	—	—	—	—	—	5	345,000

MILK PRODUCED UNDER DIRTY CONDITIONS; COWS DIRTY; UTENSILS NOT STERILIZED.

Sample No.	Sporogenes test in sets of 10 tubes, with 10 c. c. of milk.										Positive tests.	Bacterial count.
1	+	+	+	+	+	+	+	—	—	—	7	33,000
2	+	+	+	—	—	—	—	—	—	—	3	46,000
3	+	+	+	+	+	+	—	—	—	—	6	101,000
4	+	+	—	—	—	—	—	—	—	—	2	106,000
5	+	+	+	+	+	+	—	—	—	—	6	107,000
6	+	+	+	—	—	—	—	—	—	—	3	112,000
7	+	+	+	+	+	+	+	—	—	—	7	118,000
8	+	—	—	—	—	—	—	—	—	—	1	129,000
9	+	+	—	—	—	—	—	—	—	—	2	245,000
10	+	+	+	+	+	+	+	+	+	—	9	320,000
11	+	+	+	+	+	+	—	—	—	—	6	750,000
12	+	+	—	—	—	—	—	—	—	—	2	1,340,000
13	+	+	+	+	+	+	+	—	—	—	7	1,810,000
14	+	+	+	+	+	+	+	—	—	—	7	2,900,000
15	+	+	+	—	—	—	—	—	—	—	3	11,600,000
16	+	+	+	+	+	+	—	—	—	—	6	22,500,000
17	+	+	+	+	+	+	+	+	+	+	10	25,000,000
18	+	+	+	+	+	+	—	—	—	—	6	61,000,000

If the sporogenes test is any criterion, it seems evident that the pasteurized milk for the most part was not produced under very clean conditions. It would appear that in general the conditions of production were probably similar to those under which the samples represented at the bottom of the table were produced.

While information as to the production, indicated by the sporogenes test, could not be relied upon to do more than suggest probable conditions, the test might profitably be applied occasionally to milk from individual farms in connection with control work.

THE SOURCE OF THE MAJORITY OF SPORES OF B. ENTERITIDIS SPOROGENES FOUND IN MILK.

It is known that the spores of *B. enteritidis sporogenes* are widely distributed in nature. As stated, they are found in cow manure, cattle feed, soil, and water. In fact, they are so generally distributed that their presence in milk may be interpreted theoretically as contamination from a number of sources. Perhaps they come from various sources, but since they are present in manure in larger numbers than in other material likely to serve as a source of contamination, it seems logical to assume that most of them come from that source.

Race (10) believes, however, that in practice milk cans form a most fruitful source of these organisms. He offers no figures to support his belief, which, if true, naturally would decrease the value of the test as a means of detecting manurial contamination. While contamination by dirty utensils is extremely important from the standpoint of number of bacteria introduced, it is not usually of so serious a nature as that of cow manure. In the consideration of the sporogenes test the influence of utensils, however, can not be overlooked, and a number of samples were run to determine the importance of this factor.

The sporogenes test, using 10 tubes with 20 c. c. of milk, was run on samples of milk produced under dirty conditions with unsterilized utensils. A similar number of tests were then made on milk produced under the same conditions, but with sterilized utensils. Then a third set of tests was made on milk produced from cows which had been cleaned and which were kept clean, but for which the utensils were not sterilized. In fact, the utensils were simply washed with cold water. Small-top pails were used when the cows were clean, in order to exclude as much manure as possible.

From the results of this work, which are shown in Table 6, it will be seen that, taking the samples as a whole, there was very little difference in the sporogenes test between samples produced under dirty conditions with the utensils not sterilized and those produced under dirty conditions with sterilized utensils. It would seem from these results that unsterilized utensils do not contribute to any marked extent to the contamination of milk by spores of *B. enteritidis sporogenes*.

TABLE 6.—*Relation of the sporogenes test to milk produced under varying conditions.*

MILK PRODUCED IN DIRTY BARN; COWS DIRTY; UTENSILS NOT STERILIZED.

Sample No.	Sporogenes test in sets of 10 tubes, with 20 c. c. of milk.										Positive tests.	Bacterial count.
1	+	+	+	+	+	+	+	+	+	−	9	33,000
2	+	+	+	+	+	+	+	−	−	−	7	46,000
3	+	+	+	+	+	+	−	−	−	−	6	101,000
4	+	+	−	−	−	−	−	−	−	−	2	106,000
5	+	+	+	+	+	+	+	+	+	−	9	107,000
6	+	+	+	+	+	−	−	−	−	−	5	112,000
7	+	+	+	+	+	+	+	+	+	−	9	118,000
8	+	+	+	−	−	−	−	−	−	−	3	129,000
9	+	+	+	+	−	−	−	−	−	−	4	245,000
10	+	+	+	+	+	+	+	+	+	+	10	320,000
11	+	+	+	+	−	−	−	−	−	−	4	750,000
12	+	+	+	−	−	−	−	−	−	−	3	1,340,000
13	+	+	+	+	+	+	+	+	+	+	10	1,810,000
14	+	+	+	+	+	+	+	−	−	−	7	2,900,000
15	+	+	+	+	+	+	−	−	−	−	6	11,600,000
16	+	+	+	+	+	+	+	−	−	−	7	22,500,000
17	+	+	+	+	+	+	+	+	+	+	10	25,000,000
18	+	+	+	+	+	+	−	−	−	−	6	61,000,000

SAME AS ABOVE, EXCEPT UTENSILS STERILIZED.

Sample No.	Sporogenes test in sets of 10 tubes, with 20 c. c. of milk.										Positive tests.	Bacterial count.
1	+	+	+	+	+	+	+	+	+	+	10	19,000
2	+	+	+	+	+	+	+	−	−	−	7	26,000
3	+	+	+	+	+	+	+	+	+	−	9	32,000
4	+	+	+	−	−	−	−	−	−	−	3	37,000
5	+	+	+	+	+	+	+	+	−	−	8	41,000
6	+	+	−	−	−	−	−	−	−	−	2	56,000
7	−	−	−	−	−	−	−	−	−	−	0	64,000
8	+	+	+	+	+	+	+	+	+	+	10	68,000
9	+	+	+	+	−	−	−	−	−	−	4	76,000
10	+	+	+	+	+	+	+	+	+	+	10	102,000
11	+	+	+	+	+	+	−	−	−	−	6	146,000
12	+	+	+	+	−	−	−	−	−	−	4	163,000
13	+	+	+	−	−	−	−	−	−	−	3	168,000
14	+	+	+	+	−	−	−	−	−	−	4	182,000
15	+	+	+	+	+	+	+	+	−	−	8	186,000
16	+	−	−	−	−	−	−	−	−	−	1	242,000
17	+	+	+	+	+	+	+	−	−	−	7	245,000
18	+	+	+	+	+	+	+	+	−	−	8	560,000

BARN FLOOR CLEAN; COWS CLEAN; UTENSILS NOT STERILIZED.

Sample No.	Sporogenes test in sets of 10 tubes, with 20 c. c. of milk.										Positive tests.	Bacterial count.
1	+	−	−	−	−	−	−	−	−	−	1	1,310,000
2	+	+	+	−	−	−	−	−	−	−	3	1,170,000
3	−	−	−	−	−	−	−	−	−	−	0	4,200,000
4	−	−	−	−	−	−	−	−	−	−	0	4,300,000
5	+	−	−	−	−	−	−	−	−	−	1	4,400,000
6	+	+	+	+	+	+	+	+	−	−	8	4,500,000
7	−	−	−	−	−	−	−	−	−	−	0	4,800,000
8	+	−	−	−	−	−	−	−	−	−	1	7,600,000
9	+	−	−	−	−	−	−	−	−	−	1	8,400,000
10	+	−	−	−	−	−	−	−	−	−	1	9,200,000
11	+	+	+	+	−	−	−	−	−	−	4	13,600,000
12	+	+	+	+	+	−	−	−	−	−	5	18,900,000
13	+	−	−	−	−	−	−	−	−	−	1	23,900,000
14	+	+	−	−	−	−	−	−	−	−	2	32,800,000
15	+	−	−	−	−	−	−	−	−	−	1	38,000,000
16	−	−	−	−	−	−	−	−	−	−	0	64,000,000

The results of the sporogenes test with milk produced from clean cows but with dirty utensils tend to confirm this opinion. It will be noted that there was a decided drop in the number of positive tubes in the sporogenes test, although occasional samples did show a rather large number.

The quantity of sediment from each pint of milk produced under these conditions is shown in Plates VI, VII, and VIII. A comparison of the disks on Plates VI and VII shows that the milk produced under dirty conditions with unsterilized utensils and that produced under the same conditions with sterilized utensils, contained about the same quantity of cow manure. As stated, these samples showed about the same results with the sporogenes test.

When the cows were clean there was a decided drop in the quantity of manure found in the milk, as will be noted from the sediment disks in Plate VIII. Reference again to Table 6 shows that under these conditions there was a decided drop in the sporogenes test, although the utensils used in the production of this milk were decidedly dirty. The extent of the contamination which came from these dirty utensils is shown by the remarkably high counts with fresh milk in the lower section of Table 6. From these results it seems evident that, generally speaking, most of the spores of *B. enteritidis sporogenes* which are found in milk are introduced through contamination by cow manure, but that occasionally a high sporogenes test may be the result of contamination by this organism from dirty utensils.

Before considering the value of the sporogenes test any further it seems advisable to revert again to the subject of the lack of correlation between the test and the quantity of cow manure in milk. It has been shown that the test, as carried out by Savage and by Weinzirl, bore no definite relation to the sediment which may be assumed is largely cow manure. In Plates VI, VII, and VIII the results of the sporogenes test are placed in the upper right-hand corner of each square containing a sediment disk, and the total count is shown beneath the disk. Particular attention is called to Plate VII. This shows sediment disks from milk produced under dirty conditions but with sterilized utensils. If there is any positive correlation between the test and cow manure it should show with milk produced under these conditions. In making the test 10 tubes were used, each containing 20 c. c. of milk.

It is evident that with these samples and with this method there was again no definite relation between the test and the manurial content of the milk. Sample 1, for example, showed negative sporogenes test and evidently contained, from the appearance of the disk, as much manure as Samples 14, 15, and 16, in which all 10 tubes were positive.

While there seems to be no definite relation between the sporogenes test and the sediment test when individual samples under a given condition of production are compared, there seems to be a general difference in results when a number of samples produced under dirty conditions are compared with those produced under clean con-

ditions. This has been emphasized in the discussion of the test in connection with certified milk and dirty milk, and is again illustrated by the results shown in the last two sections of Table 6.

SUMMARY AND CONCLUSIONS.

1. The Savage test using 10 tubes with 2 c. c. of milk is not sufficiently delicate to be of great value, evidently, because not enough milk is used in each tube.

2. The Weinzirl test does not appear to correlate with the quantity of manure in milk. This seems to be due both to the method of making the test and to the variation in spore content of *B. enteritidis sporogenes* in manure.

3. The experiments indicate that the majority of spores of *B. enteritidis sporogenes* gain entrance to milk by means of cow manure.

4. With 10 tubes and 20 c. c. of milk to each tube, the sporogenes test as used throughout the experiments shows a fairly definite relation to conditions of production. This relation is more definite than that shown by either the Savage or the Weinzirl test.

None of the tests, however, show a definite correlation between the number of tubes positive and the quantity of manure in any given sample. But with 20 c. c. quantities of milk and discounting individual variation, there is a general trend of agreement between the test and manure.

5. As a rule, the sporogenes test with 10 tubes and 20 c. c. of milk shows high results, that is, a large number of positive tubes with milk produced under dirty conditions. With milk produced under clean conditions, the test is apt to be negative or show only a few positive reactions.

The test tends to differentiate between extremes in methods of production, and naturally most milk will fall between these limits. The nearer the conditions of production approach one extreme or the other, the more accurately will the sporogenes test indicate the conditions.

It must be pointed out, however, that no reliance can be placed on one test with one sample. Individual variation with a single test makes a series of tests on a number of samples necessary for the result to have any significance.

6. When the limitations of the test are understood and the results properly interpreted, its use with a series of samples from a given source should give considerable information as to cleanliness of production, particularly in connection with the extent of manurial pollution. The test, if used, however, should be taken as merely an indication of the conditions of production, and should be verified by an actual inspection at the farm.

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